THE EFFECT OF INGESTED RADIOPHOSPHORUS ON EGG PRODUCTION AND EMBRYO SURVIVAL IN THE WASP HABROBRACON [1, 2, 3]

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The present report is the first of a series concerned with investigations of the developmental and genetic effects of ingested radioactive isotopes. Although an appreciable amount of information is becoming available on the vertebrate histopathology of internal radioactive isotopes, apparently there have been few studies directed toward the cytogenetic consequences. Even in such organisms as insects, which produce large samples of offspring in a relatively short time, available data are limited to a few short papers on Drosophila (King, 1949; Bateman and Sinclair, 1950; Blumel, 1950).

The insect chosen for present investigations, an ectoparasite of Ephestia caterpillars known in genetic literature as *Habrobracon juglandis* (Ashmead), has the Hymenopteran advantage for induction experiments of parthenogenetically produced, genetically haploid males. Thus the haploid egg and the organism developing from it can indicate the effects of experimental treatment without the complications of another set of chromosomes. As will be detailed in "Materials and Methods," particular advantages were discovered in the use of Habrobracon for feeding experiments.

The purpose of the following experiments was to evaluate among embryonic offspring the total induced lethality, including recessive lethals, dominant lethals and non-nuclear damage, for a series of dosages ranging from tracer dilutions up into the intermediate category of radioactivity encountered in medical therapy (Levy, 1946). Phosphorus, the element chosen, is well represented in nucleic acids which are intimately associated with the structural elements of cell nuclei. Incidental observations on the longevity of treated animals are included, which indicate a remarkable radio-resistance of adult wasps.

Materials and Methods

The Habrobracon stock used was wild type number 33 known at least 15 years for its high hatchability (Whiting, 1940). Unmated females were stored in clean shell vials (70 × 20 mm.) for five days in an incubator at 30° C. Such conditions

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of starvation produce a hungry individual with an abdomen very obviously flattened dorsoventrally. The visible change in abdominal shape reflects a utilization of reserves stored in the fat body and the disappearance of all but a few full-sized uterine eggs (Grosch, 1950). At the end of the fifth day a droplet of white clover honey with or without P^{32} was delivered to the wall of the vial from the end of a straightened No. 3 paper clip. Typically the hungry wasp is attracted to the honey within one minute, and, standing upon clean surface, contacts the honey only with the mouth parts. This is a distinct advantage over experimenting with insects which move over the surface of a radioactive medium when feeding. The gonads of an insect with such "browsing" habits are constantly near an external source of irradiation and the interpretation of results is thereby complicated. We believe that ingestion experiments with Habrobracon most nearly approach an evaluation of the effects of internal radiation alone. Note further that only one feeding was used per female.

During feeding, the flattened abdomen gradually swells until its fullness approaches that of an unstarved wasp. The re-attained rotundity is due entirely to the filling of an expansible crop situated in the anterior of the abdomen. This is a second advantage, because by mere external inspection the actuality and degree of feeding can be determined.

The braconid digestive tract extends in direct course from mouth to anus with the crop an enlarged, thin-walled connection between the esophagus and stomach. It is the structure known in the honey bee as the honey stomach. It is not a diverticulum as in other orders of insects and within a day its contents are moved into the midgut, yet the crop with its strong anterior and posterior valves can serve in efficient immediate storage of a significant volume of ingested food (Grosch, 1951). The capacity of the crop furnishes a defined volume basis for dosage in feeding experiments (a third advantage). The mean volume for distended crops from undwarfed adult females was determined as 0.49 ± 0.06 mm^3.

When the crop is completely filled, the wasp loses interest in feeding. The majority of Habrobracon females attain satiety at one continuous feeding although a few individuals seem to prefer intermittent ingestion. Because of the latter type of behavior, the standard procedure adopted was to allow a given female one hour in the vial with the food droplet. At the end of such a period the great majority of wasps are completely fed. This was determined by an inspection of the degree of distension of the abdomen.

Twelve hours after the single honey feeding each female was given two pre-stung caterpillars on which to deposit eggs. Stender dishes (60×35 mm.) large enough for spreading out the 10 caterpillars parasitized by 5 females were the containers used at this phase. Caterpillars rested upon white paper fitted into the bottom of the dishes. A daily renewal of one caterpillar per female has been adopted as the standard procedure. If additional caterpillars appeared discolored or unduly flaccid, they were replaced and a notation made on the data sheet. Such precautions are believed necessary to avoid introducing variables due to the condition of the host.

Eggs were collected every 24 hours and immersed in mineral oil (Nujol) contained in Syracuse watch glasses, a procedure determined as giving hatchability records even better than eggs left on the host. After 48 hours incubation in mineral oil at 30° C., the number of larvae which had hatched was counted and recorded. In our experience, since 30 hours is the normal hatching time at 30° C., all larvae which can emerge will have done so in 48 hours.

In all but the first experiment a weight basis was used for the preparation of samples of honey containing P^{32} at various activities. To this end, small (36 × 12 mm.), thick-walled bottles equipped with plastic screw caps were weighed before and after adding pipetted amounts of honey and diluted radioactive phosphorus in the approximate proportions needed to obtain a desired activity. In setting up the initial experiment it had been discovered that the forces of adhesion and cohesion negated pipetted delivery of exact volumes of honey. Dosages mentioned below are given in terms of the activity at the time of ingestion. The complicated question of internal dosage, although under consideration, has not progressed to a state which would warrant usage in the present paper.

Records upon the laying and hatchability were taken for 42,832 eggs, of which 24,832 were controls. Table I presents a numerical summary of experiments to indicate their scope and sample size. Also shown is the number of females used in each experiment. The analysis presented in the section on "Results" is a consideration on an average-per-animal basis.

TABLE I

A summary indicating the size of ovipositing groups and the total number of eggs laid by each group

Experiment number	Experimentals			Controls	
	Activity of material ingested in μc/g.	Number of females	Eggs laid	Number of females	Eggs laid
5	1,445	22	498	19	3,496
6	1,070	22	604	19	3,010
1	1,000−	19	769	21	3,379
2	271	17	816	18	2,377
3	166	20	2,671	20	3,152
4	43	16	2,471	17	1,820
7	20.6	20	2,264	17	2,684*
9	16.99	24	4,640	24	4,914
8	12.6	16	2,854	17	2,684*
		176	17,587	155	24,832

* The same control was used for two simultaneous experiments, numbers 7 and 8.

RESULTS

The gametic sequelae of treatment internally with P^{32} were found to have organization on the basis of the treatment's influence upon egg production. That is, egg laying (1) may or (2) may not be halted comparatively early in the life of a treated female. A dose adequate to halt egg production is about 200 μc/g. At lower doses, egg production continues throughout the life of the female. However, two types of effects are observed: (a) Between doses of 50 to 200 μc/g. there is definite falling off in egg production followed by recovery; while (b) below 50 μc/g. no statistically significant decrease in egg production is demonstrable.

(1) *Egg laying halted.* Figure 1 depicts the average day by day egg production for females fed sufficient radioactive phosphorus to cause early cessation of egg laying. Presumably at massive dosages, still fewer eggs would appear in an

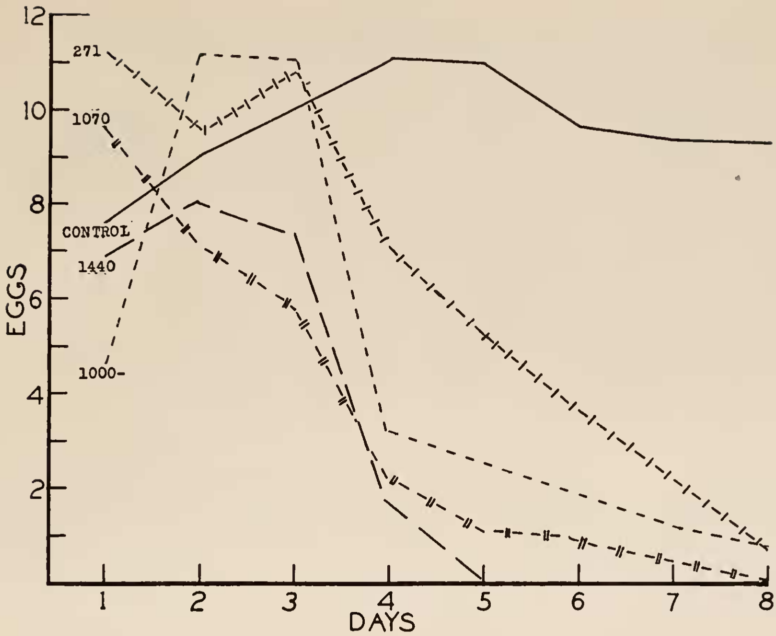

FIGURE 1. A comparison with combined controls of the average number of eggs produced per female per day by Habrobracon which ingested radioactive phosphorus sufficient to halt egg production. The low number of eggs produced in the first day of the 1000-experiment is non-significant; the individual control showed a similar value. The designation at the beginning of each curve is the activity in millicuries per gram of the mixture fed.

approach to the limit of those eggs differentiated before treatment and un-resorbed during a five day starvation period. On the present graph an orderly relation is noted with the highest dosage causing earliest discontinuation of egg laying and the least number of eggs laid. At successively lower dosages a sequence in number

TABLE II

Cumulative total of average number of eggs produced per female

Experiment number	Activity of mixture ingested in μc/g.	Day 1	Day 4	Day 9	Day 19	Day 29	Day of death
5	1,445	7	23				23
6	1,070	9	26	28			28
1	1,000−	5	30	36			36
2	271	11	39	50			50
3	166	10	47	82	103	169	206
4	43	7	31	74	126	190	192
7	20.6	3	27	64	91	140	158
9	16.99	9	36	76	174	258	269
8	12.6	2	29	67	153	226	249
Controls	0	8	38	85	161	217	238

TABLE III

Average number of eggs produced per female during designated periods

Experiment number	Activity of material ingested in μc/g.	Days					
		1	2–4	5–9	10–19	20–29	29–death
5	1,445	7	16				
6	1,070	9	17	2			
1	1,000–	5	25	6			
2	271	11	28	11			
3	166	10	37	35	21	66	37
4	43	7	24	43	52	64	2
7	20.6	3	24	37	27	49	18
9	16.99	9	27	40	98	84	11
8	12.6	2	27	38	86	73	23
Controls	0	8	30	47	76	56	21

of eggs laid is seen corresponding to the dosage of radioactive phosphorus ingested. Tables II and III give a numerical synopsis of these facts.

On Figure 2 is plotted the percentage of the Figure 1 eggs which hatched. In general, the higher the dosage of ingested radioactive phosphorus, the lower is the hatchability. An inspection of Table IV, which summarizes hatchability in periods

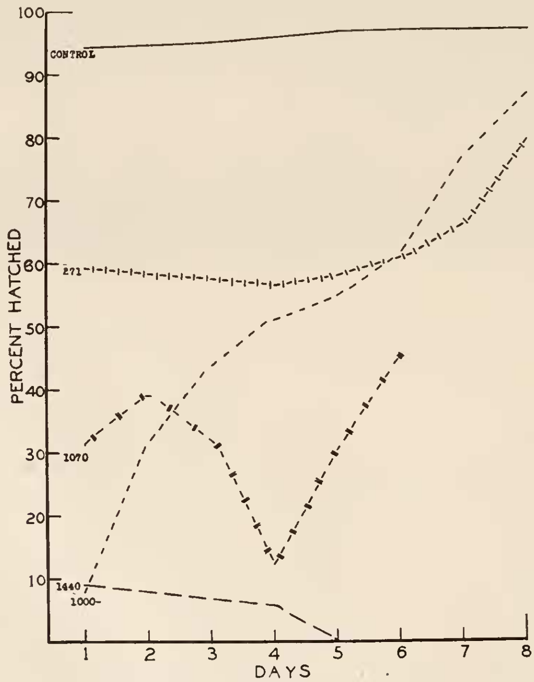

FIGURE 2. The hatchability of the eggs of Figure 1. The activity in millicuries per gram of the mixture fed is again indicated at the beginning of each curve.

TABLE IV

Average percentage of hatchability of eggs laid during designated periods

Experiment number	Activity of material ingested in μc/g.	Days					
		1	2–4	5–9	10–19	20–29	29–death
5	1,445	9	7				
6	1,070	9	22	28			
1	1,000−	8	44	67			
2	271	60	58	67			
3	166	93	87	85	52	53	63
4	43	98	97	96	95	80	50
7	20.6	51	83	84	75	57	45
9	16.99	96	97	96	94	87	—
8	12.6	59	91	94	91	80	62

selected on the basis of all primary curves (Figs. 1, 2, 3 and 4), demonstrates the hatchability relationship especially well in the 2 to 4 day period. In those experiments in which eggs were produced several days beyond the fourth day, there is shown a rise in hatchability for the last groups of eggs laid. However, it should

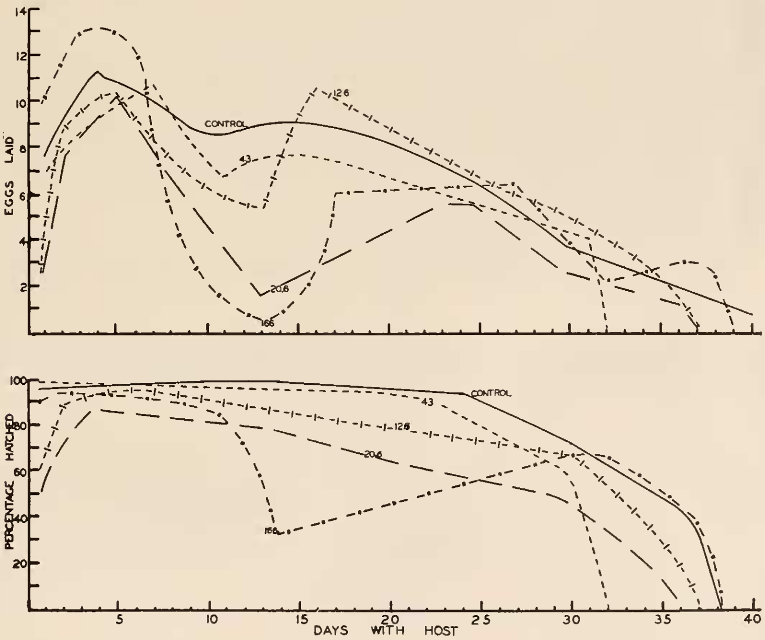

FIGURE 3. A comparison with combined controls of the eggs laid per female per day (above) and the hatchability thereof (below) in experiments when eggs are produced throughout the life of treated females. Curves are smoothed after the thirtieth day. The activity in millicuries per gram of the mixture fed is indicated along the middle of each curve where space is more adequate rather than at the beginning of the curve when the activity was at the level designated.

be remembered that these samples are quite small and the viability of even a single egg markedly affects the numerical result.

(2) *Egg laying not halted.* Figures 3 and 4 depict eggs laid and the hatchability thereof for experiments in which egg production continued throughout life. The results plotted in Figure 4 were treated separately to avoid adding confusion to already complex drawings (Fig. 3). Furthermore, unlike all other experiments, in the investigations for Figure 4, egg laying and hatchability were not recorded after the thirty-first day of oviposition. (In actuality, records beyond the thirty-first day would have been insignificant additions to the data obtained since only one animal survived the thirty-second day.)

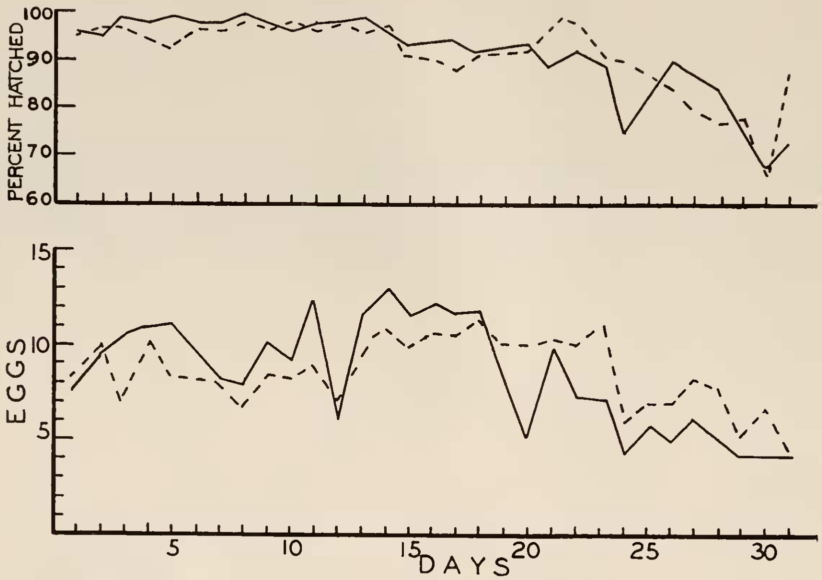

FIGURE 4. The eggs produced (below) and the hatchability thereof (above) in experiment No. 9. The solid lines represent control values. The broken lines represent results on an average per animal basis for feeding with a honey mixture at a radioactivity of 16.99 millicuries per gram.

In general, egg production both in controls and in experimentals is characterized by a peak between days 4 and 9 followed by a low between days 9 and 19. Following this low, the number of eggs laid per day becomes high again until about day 29. However, except for one experimental case to be discussed, egg production does not recover to the level of that during days 4 to 9. During the senescent period, the final days of life after the thirtieth day, there is a gradual dropping off in eggs produced.

As is shown in Table II higher doses tend to result in fewer eggs. When plotted graphically this consequence of treatment is not strikingly evident except throughout the course of days 9 to 19. During this period the drop in egg production is much accentuated in higher dosages. However, after day 19 the production of eggs by treated and untreated wasps becomes similar so that curves approach each other and differences are not significant. Table III furnishes a numerical summary by designated periods of eggs laid.

The results observed when the number of emerged larvae is compared with the number of eggs laid are rendered in percentages and plotted in Figures 3 and 4. The resultant curves are not as complex as those for egg production. Except for the highest dosage in this group (166 μc/g.) no obvious depression was demonstrated in the graphs.

In general, until day 29, the experimental curves parallel the curve for controls but at a lower value indicating a measure of lethality. After the twenty-ninth day the differences between controls and experimentals are statistically non-significant. In fact, in unsmoothed plots from raw data the lines cross and recross almost at daily intervals. Again it should be remembered that the samples of eggs obtained become small in the last few days.

Survival curves

A popular method of representing the results of radiation experiments is by means of the survival curves obtained through plotting the proportion of organisms surviving against the dose. This gives a picture of the manner in which the number of organisms affected increases with the dosage of radiation. In the present case, the values of the first three designated periods shown in Table IV have been used as a basis for Figure 5. The curves obtained promise to be particularly useful by indicating ranges of irradiation in which it would be interesting to have additional data. The curves indicate a more complicated type of action than the geometric progression (exponential curve) of a system involving single ionizations of targets (Lea, 1947). This was expected in view of the inclusive nature of induced lethality in the present experiments. On the other hand, it was interesting to find that our curves for first day egg hatchability exhibit a shape similar to that obtained when the emergence of adult Drosophila is plotted against the initial activity of P^{32} added to culture medium (Arnason, Irwin and Spinks, 1949).

Longevity

By calculating the means and their standard errors, a conception is obtained of the survival time of treated and untreated females. These results are presented in Table V, calculated as days after emergence from cocoons. To obtain days after

TABLE V

Mean longevity in days of females which have ingested P^{32}

Activity of material ingested in μc/g.	Experimental	Control
1,445	23.14 ± 2.08	27.89 ± 1.35
1,070	24.82 ± 1.74	25.37 ± 1.58
1,000–	21.32 ± 1.81	27.09 ± 2.47
271	25.06 ± 3.09	22.06 ± 2.31
166	26.45 ± 2.16	23.45 ± 2.02
43	26.94 ± 1.83	21.59 ± 2.11
20.6	29.10 ± 2.75	26.94 ± 2.71
16.99	27.17 ± 1.90	27.92 ± 1.85
12.6	28.63 ± 1.96	26.94 ± 2.71
		Combined controls 25.73 ± 0.65

treatment, five days should be subtracted from each value. It is evident from the data obtained that longevity of wasps was not influenced significantly by internal β radiation over the range investigated. In fact, the experimentals show higher mean survival time than the corresponding controls in five out of the nine experiments. In the distribution and range of values which make up the array from which mean survival time is calculated, examples of both treated and non-treated wasps were obtained in the long-lived category of 40 days after the honey feeding. Typically, at

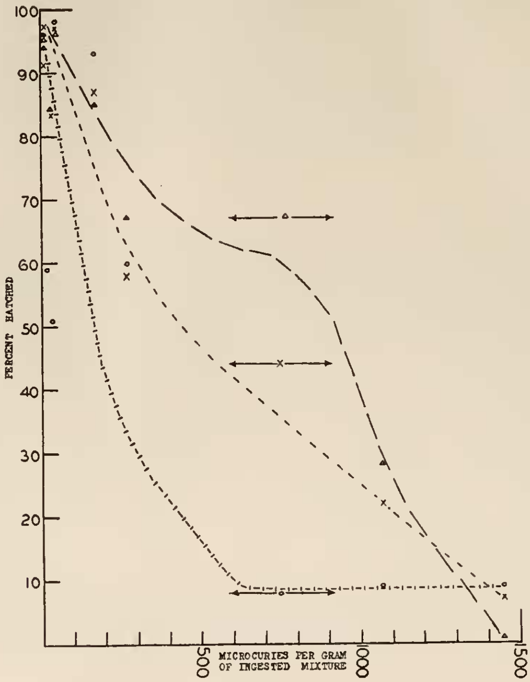

FIGURE 5. Survival curves obtained by plotting the percentage of hatched eggs against the radioactivity of the mixture ingested. The results for the first day of egg laying are designated by ovals. The corresponding curve is drawn of alternating horizontal and vertical dashes. The results for the period comprising the second to fourth day are designated by x marks and the curve is drawn using short dashes. The results of the period comprising the fifth to ninth day are designated with triangles and the curve is drawn using long dashes. The arrows mark the possible range of dosage for the initial experiment when pipetted measurement was attempted.

least three to six animals were alive after 30 days of experimental observation. Such longevity was spectacular in experiments in which egg production was halted five to eight days after treatment, yet the sterilized females remained alive for weeks after laying their last egg. A more comprehensive investigation into what appears to be remarkable radio-resistance by adult braconids is in progress.

Discussion

Interpretation of the above results is difficult because we have not been able to find comparable studies with the day-to-day life-time approach. In general the total and average egg productivity of controls compares well with the surveys of Hase (1922) and associates who obtained more than 200 eggs per individual braconid collected from nature. However, hatchability records by these European investigators run between 50 and 70 per cent whereas hatchability in the selected laboratory stock used by us and by A. R. Whiting is typically above 95 per cent. Even when the senile period is included the overall average hatchability is at least 80 per cent.

Occasional checks on egg production and hatchability at various ages have been made during exploratory and control studies by A. R. Whiting (personal communication). However these data have not been published nor do they include observations during the senile period. In the published studies with unmated females (Whiting, 1940, 1945) attention is directed chiefly to the first period of oviposition in the interest of comparing the radio-sensitivity of eggs in successive stages of first meiotic pro- and metaphase. Habrobracon furnishes ideal material for such studies and the results throw some light upon certain of our findings.

The braconid ovary is comprised of four ovarioles of the polytrophic type, thus containing alternating successions of oocytes and trophocytes for at least three quarters of each tube's length. The anterior end of each ovariole is made up of mitotically active oogonia subsequently differentiated into cell groups, of which the most posterior cell becomes the functional oocyte while remaining members of the group become trophocytes. As an oocyte is moved posteriorly its nucleus passes through prophase of first maturation division; eggs ready to be deposited are stored in the posterior of each ovariole in first meiotic metaphase. These observations from dissections were correlated with the results of X-radiation experiments (Whiting, 1940) to demonstrate that eggs in first meiotic metaphase were relatively susceptible to radiation damage, whereas oocytes in prophase with diffuse chromatin were relatively resistant. Radio-sensitivity and radio-resistance were reflected by low hatchability and high hatchability, respectively. Such differences in sensitivity could explain the rise in hatchability shown by the present curves during days 6 to 8 wherein the greater percentages hatched of the eggs laid on these days could reflect a resistance of prophase eggs to radiation.

It is not settled whether most of the radiation damage occurs shortly after ingestion or if injury occurs during an appreciable number of successive days. At present we lean toward the former, since it is suspected that much radioactive phosphorus is lost by incorporation into the eggs laid. Although radio-autography of Habrobracon has not yet been performed, it is well established in biological material that the deposition of P^{32} is heaviest in regions of the greatest metabolic and mitotic behavior associated with growth and differentiation. Typically, in the adult holometabolous insect, cell division and differentiation take place only in the gonads. Thus, Irwin, Spinks and Arnason (1950) find that Drosophila gonads continue to show very high P^{32} content in the adult when general growth of all other tissues has ceased. Consistent with these concepts is the present fact that with Habrobracon egg laying prolonged throughout life, experimental results in both egg production and in egg viability ultimately approach control values.

There are certain differences between our results and those of A. R. Whiting especially noticeable in the first day of egg laying on which the number of eggs obtained by us is consistently lower. This is undoubtedly due to the differences in the conditions of the experiment. The animals of the present experiment were maintained under circumstances which prevent the formation of uterine eggs, whereas the conditions in the Whiting experiments were conducive to the accumulation of uterine eggs.

Our finding a cessation of egg production after subjecting females to higher dosages of internal radiation seems another addition to the several reports of the radio-sensitivity of oogonia in various animals. Four examples have come to our attention: (1) With higher X-ray dosages Whiting (1940) discovered oogonial regions vacuolated, fragile and with pyknotic nuclei attendant to a falling-off in Habrobracon egg production. (2) A case of total absence of ovaries has been reported for female mosquitoes raised in baths containing P^{32} at activities of around 200 mμc. (Bugher and Taylor, 1949). (3) The most spectacular effect of internal radiations in vertebrates is shown in mice injected with P^{32} by reduction in the size of ovaries (Warren, MacMillan and Dixon, 1950). The immediate size reduction following injection is due to degenerative follicular changes; however, a failure to regain normal size is traceable to subsequent greatly diminished oogenesis. (4) Ionizing radiations are found to exert a selective and destructive action on the gonads of round worms (Alicata, 1951). These representative cases from such diverse phyla suggest a universal consequence to be expected in radiation experiments. It seems likely that numerous other examples may exist in published and unpublished data on still other types of animals.

When our observations on unhalted egg laying are considered, for comparison are available the lifetime records of egg laying for birds, especially chickens (Hutt, 1949), and for other insects, especially Drosophila (Gowen and Johnson, 1946). Records for all forms of life show a decline in egg production with age as do the present data. In this effect of senescence, as might be expected, the abilities of Habrobracon more nearly resemble those obtained for dipteran rather than those obtained for avian egg production. This is explainable on the basis that the normal egg production by insects is not markedly influenced by cyclic events. Egg laying as plotted by us for Habrobracon shows smoother day-to-day curves for the first half of the wasp life period than exhibited by eight different races of fruit flies considered by Gowen and Johnson. However, by suggesting two peaks, our curves seem more complicated in their general shape. A current experiment is being performed in an attempt to estimate whether starvation conditions preceding egg laying complicate the egg production curve. If not, an alternative explanation may be based on the change-over from eggs produced by oocytes differentiated before eclosion to eggs produced by oocytes differentiated during the laying period.

Lifetime records of modifications to egg laying, due to experimental effects comparable to ours, have not been discovered in available literature. In order to obtain data somewhat comparable, we have gone to reports concerning the effects of ionizing radiations on cell production, where we find curves which ours resemble. This is so for diagrammatic presentations of the rate of change in peripheral blood cells following graduated dosages of penetrating radiations (Cronkite, 1949). Blood cells are the ultimate products of mitotically active tissues. In turn, this thought suggested another source of comparable curves, those based upon mitotic index. Here, too, we note a resemblance, especially in the shapes of our lines plotted for the period between days 9 and 29 (Fig. 4). These curves are interestingly reminiscent of certain results obtained by Canti and Spear (1929) in irradiated, cultured chick tissue. Similar consequences of radiation have been demonstrated in other rapidly dividing tissues from such diverse origins as beans, rats and tadpoles (Lea, 1947). As measured by division activity, higher dosages result in a drop in cell production with the control level never regained, whereas in intermediate dosages a recovery wave is obtained which exceeds the control level. A present example of a recovery wave exceeding the control may be furnished by the 12.6 μc/g. curve.

An accepted explanation of such recovery waves is that the mode results when two classes of cells arrive at the same stage at a given time. A class of cells not in the susceptible phase at the height of treatment, and hence not greatly influenced, is supplemented by another class of cells which were delayed in division but have recovered. In explanation of our lifelong egg production curves we postulate similar delayed division effects upon the oogonia. It is conceivable that the oogonia forming a mitotically active cell mass would have cells in susceptible phase at the height of the treatment, which could be delayed in division and development.

Summary

1. Changes in the eggs produced per day and in the hatchability thereof are reported for virgin Habrobracon fed honey containing P^{32} at radioactivities ranging from the tracer level into the intermediate level of internal therapy (10 μc to 1500 μc).

2. Above 200 μc/gm. of ingested mixture, egg laying ceased after a period of a few days corresponding to a fraction of the supply of unresorbed ova and oocytes differentiated before treatment. The viability of the embryos from these eggs, as measured by the number hatched, is correlated with the level of activity in the material ingested. A rise in viability in the seventh to eighth day, in experiments in which egg production continued that long, is interpreted as an indication of the lesser susceptibility of prophase eggs.

3. Below 200 μc/gm. of ingested mixture, egg laying continued throughout the life of the treated females; however, with higher dosages fewer eggs were produced. This is traceable chiefly to a decline in the eggs laid daily during the middle period of life. Curves obtained show recovery waves reminiscent of those found for cell production from rapidly dividing tissues after irradiation. Viability of the eggs produced during the first two-thirds of life is correlated with dosage. During the senescent decline in egg production, typical of both controls and experimentals, no significant differences in hatchability were obtained.

4. A remarkable radio-resistance was discovered, in that treated animals in *all* experiments lived as long as or longer than controls.

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